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Regulatory functions of alloreactive Th2 clones in human renal transplant recipients

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Regulatory functions of alloreactive Th2 clones in human renal transplant recipients.

Background. Chronic allograft rejection is the major clinical problem in organ transplantation. There is evidence that indirect T cell recognition of donor-specific HLA peptides may play an important role in the immunopathogenesis of chronic allograft rejection. We have recently shown that HLA allopeptide-specific T cell clones generated from renal transplant recipients with chronic allograft nephropathy are of the Th1 phenotype, while those from stable patients are Th2. There is evidence in experimental animal models of autoimmunity and transplantation that Th2 cells may function to regulate immune responses, but the biological relevance of these observations in humans has not been reported.

Methods. The purpose of this study was to investigate the putative regulatory functions of alloreactive human Th2 clones. HLA-DR allopeptide-specific Th1 and Th2 cell clones were generated from peripheral blood lymphocytes of human renal allograft recipients with chronic allograft nephropathy (CAN) or with stable renal function (SRF), respectively.

Results. An in vitro co-culture system showed that the proliferative responses of Th1 clones from patients with CAN were significantly inhibited by the Th2 clones in response to the donor-derived HLA allopeptides. In addition, co-culture of the Th2 clones inhibited cytokine production (IFN-γ) by the Th1 clones in response to the donor-specific peptides. The regulatory functions of Th2 clones were antigen-specific since they only occurred when both the Th1 and Th2 clones were reactive to the same HLA-DR allopeptide, and were mediated by IL-4 and IL-10.

Conclusions. This is the first demonstration, to our knowledge, indicating that Th2 cells may function to regulate indirect Th1 alloimmune responses that are critical for the progression of CAN in humans.

Key words: regulatory cells, T cell lines, cell clones, cytokines, renal transplantation, chronic allograft nephropathy, peptides, HLA, organ rejection.

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T cell recognition of alloantigen is the key primary event that initiates allograft rejection, although several other factors may contribute to acute and chronic allograft dysfunction [1–5]. T cells recognize alloantigen via two distinct yet non-mutually exclusive pathways: the direct and indirect pathways of allorecognition [2, 6]. In the direct pathway, alloreactive T cells recognize intact allo-major histocompatibility complex (MHC) molecules on the surface of donor cells, while in the indirect pathway, T cells recognize alloantigens in the form of peptides after processing and presentation by self-antigen presenting cells (APC's). Experimental studies have shown that both pathways could mediate skin allograft rejection [7, 8]. However, it has been suggested that the direct pathway of allorecognition predominantly mediates acute allograft rejection, while the indirect pathway of allorecognition mediates chronic rejection of vascularized grafts [2, 3]. Recent data from our group and others in human renal, cardiac and lung allograft recipients support this hypothesis [9–13]. In experimental animals, priming via the indirect pathway promotes development of chronic allograft vasculopathy in small and large animal models [5, 14].

In a series of animal studies, we have shown that class II MHC allopeptide-specific T cell clones from rats with acute rejection produce interferon-γ (IFN-γ), whereas clones from tolerant animals produce interleukin (IL)-4 and IL-10 in response to the relevant allopeptide in vitro [15–17]. Adoptive transfer of T helper-1 (Th1) clones into naïve LEW rats induced alloantigen-specific delayed type hypersensitivity (DTH) responses, while transfer of Th2 clones did not. Co-adaptive transfer of the Th1 and Th2 clones suppressed DTH responses to the allopeptide and irradiated donor splenocytes. In vitro, irradiated Th2 clones inhibited the proliferative response of the Th1 clones and suppressed IFN-γ production. In our pilot study in human transplant recipients the HLA-DR allopeptide-specific T cell clones generated from patients

with chronic allograft nephropathy (CAN) secreted Th1 cytokines, whereas clones from patients with stable renal function (SRF) produced Th2 cytokines in response to donor-specific HLA-DR allopeptides [17]. The aim of our current study was to investigate the regulatory functions of Th2 clones generated from human renal transplant recipients, and to prove the biologic relevance of these experimental findings in animals into humans.

METHODS

Patients

The renal transplant recipients who were used to generate the T cell clones were previously described [9, 17]. Briefly, renal allograft recipients who had received their transplants at least six months earlier and were attending the renal transplant clinic at the Brigham and Women's Hospital were screened. All patients were on triple therapy immunosuppression consisting of cyclosporine, steroids, and azathioprine. Patients of specific interest had to be mismatched for one or more of the three candidate HLA-DR antigens for which synthetic peptides were available (DR1, DR2, and DR3). Two patients with biopsy-proven chronic allograft nephropathy with an elevated serum creatinine level (>2.0 mg/dL) were compared with three patients with stable allograft function (stable serum creatinine <2.0 mg/dL). The study was approved by the human subjects committee of the Brigham and Women's Hospital.

Peptides

A panel of peptides corresponding to the β-chain hypervariable regions of HLA-DRB*0101 (residues 6-21, 22-41, 42-62, 63-80, 81-94), HLA-DRB*1501 (residues 1-21, 22-40, 41-60, 61-80, 81-94), and HLA-DRB*0301 (residues 6-21, 22-41, 42-62, 63-80, 81-94) was synthesized (Quality Controlled Biochemicals, Hopkinton, MA, USA) as previously described [9, 17].

Generation of T cell lines and clones

The generation and characterization of the human Th1 and Th2 cell clones used in this study was previously described [17]. Two cell lines (Th1) were obtained from the patients with CAN and three cell lines (Th2) were obtained from the stable patients. A total of eleven (Th1) and twenty (Th2) T cell clones were generated, respectively, by limiting the dilution as previously described [17]. Of these, four Th1 clones with different T cell receptor phenotypes derived from one cell line and reactive to HLA-DRB*0101 (residues 42-62) were studied along with three Th2 clones reactive to the same peptide and three Th2 clones reactive to HLA-DRB*1501 (residues 41-60). Similar results were obtained in all experiments and representative data are shown from one set of studies.

Enzyme-linked immunosorbent spot (ELISPOT) assay

The ELISPOT assay was used to measure the frequency of alloreactive T cell clones producing the Th1 cytokine IFN-γ and the Th2 cytokine IL-10, as previously described [18]. Briefly, ELISPOT plates [Cellular Technology Limited (CTL), Cleveland, OH, USA] were coated with capture antibodies against INF-γ or IL-10 (Endogen, Rockford, IL, USA) in phosphate-buffered saline (PBS) and left overnight at 4°C. The plates were blocked with PBS-bovine serum albumin (BSA) 1% for one hour and then washed with PBS. A total of 5×10^5 peripheral blood leukocyte (PBL) were added to each well in 100 μL of complete RPMI medium [90% RPMI, 10% human serum (Sigma, St. Louis, MO, USA); L-glutamine and penicillin/streptomycin (BioWhittaker, Walkersville, MD, USA); 50 mmol/L 2-mercaptoethanol (Sigma)] and the relevant HLA-DR peptide. Control wells contained cells plus medium alone or APCs without the peptide. After 48 hours, the plates were washed, biotinylated detection antibodies added and the plates were left for a further overnight incubation at 4°C. After further washing, HRP conjugate (Dako, Glostrup, Denmark) was added for two hours at room temperature. Development was with AEC (Pierre Pharmaceuticals, Rockland, IL, USA; 10 mg/mL in N,N-dimethylformamide) freshly prepared in 0.1 mol/L sodium acetate buffer (pH 5.0) mixed with 30% H_2O_2 (200 μL per well). The resulting spots were counted on a computer-assisted ELISPOT Image Analyzer (CTL; Fig. 1). The results were then calculated and presented as the frequency of cytokine-producing cells per million cells.

Proliferation assay

T cell clones (2.5×10^4 cells) were cultured with 10 μg of the relevant donor mismatched HLA-DR allopeptide in the presence of irradiated APCs for 72 hours. Human neutralizing antibodies, rat anti-human IL-4 monoclonal antibodies (mAb; 0.5 to 25 μg/mL), rat anti-human IL-10 mAb (0.5 to 25 μg/mL), or rat IgG1 isotope control antibody (all from PharMingen, San Diego, CA, USA) were added to the cultures. As the optimal effects of anti-IL-4 and anti-IL-10 mAb were observed at a concentration of 10 μg/mL, that dose was used for anti-IL-4, anti-IL-10, and isotope control antibodies in the experiments. Lymphocyte proliferation was determined by measuring the ^3H thymidine incorporation [17]. In the co-culture system, irradiated (3000 Rads) Th2 clones (2.5×10^4) were incubated with an equal number of the Th1 clones (2.5×10^4) and the relevant HLA-DR allopeptide. Background proliferation was assessed by incubating the cells with culture media alone without the peptides.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from T clones were cultured with 10 μg of donor specific allopeptide and with the neutralizing

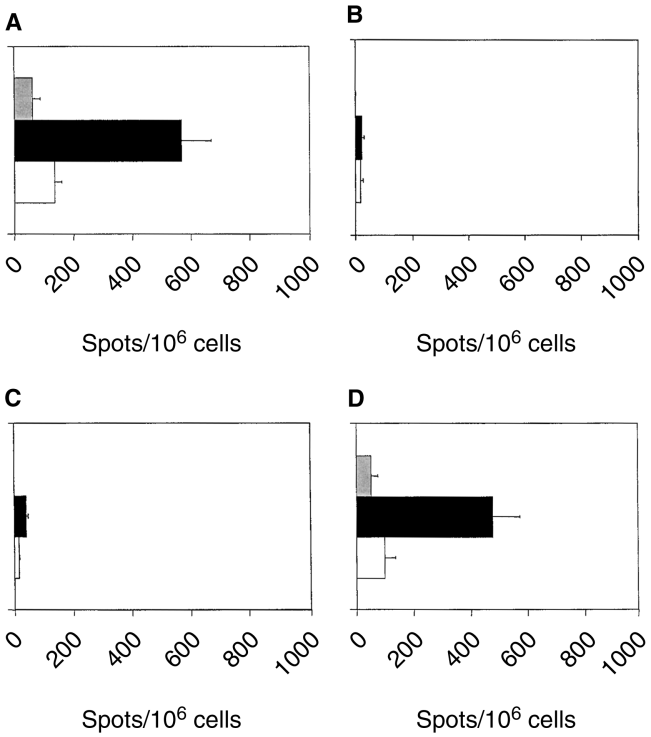

Fig. 1. ELISPOT analysis of T cell clones from renal transplant patients generated with donor specific HLA-DR allopeptides. (*A*) Interferon-γ (IFN-γ) and (*B*) interleukin (IL)-10 of a representative T cell clone from a patient with chronic rejection (Th1 clone); (*C*) IFN-γ and (*D*) IL-10 of a representative T cell clone from a patient with stable renal function (Th2 clone). Symbols are: (■) control peptide; (■) allopeptide; (□) no peptide.

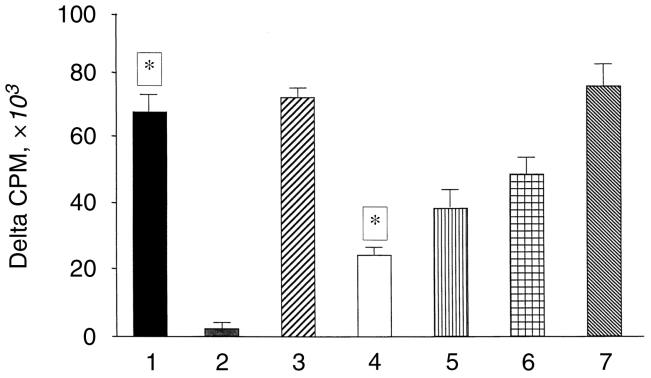

Fig. 2. Regulatory function of Th2 clones. Proliferation assay of co-culture of Th2 clones from patients with stable renal function (SRF) irradiated with 3000 rads, cultured in a 1:1 ratio with Th1 clones from chronically rejecting patients (CR). (*1*) Th1 clones specific for HLA-DR1 (42-62) + peptide HLA-DR1 (42-62). (*2*) Irradiated Th2 clones specific for HLA-DR2 (41-60) + peptide HLA-DR2 (41-60). (*3*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptide HLA-DR1 (42-62). (*4*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60). (*5*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing IL-4 mAb. (*6*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing IL-10 mAb. (*7*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing anti-IL-4 and anti-IL-10 mAb. Data are expressed as mean ± SEM; $*P < 0.0001$, 1 vs. 4; $N = 4$.

anti-IL-4 mAb, anti-IL-10 mAb, or rat IgG1 isotope control were obtained at 48 hours for measuring the level of secreted Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-10) cytokines. Cytokine production was measured by ELISA using BioSource Cytoscreen Kits (BioSource, Camarillo, CA, USA), as previously described [17].

RESULTS

In a recent study we generated T cell lines and clones from peripheral blood lymphocytes of renal transplant recipients against donor-derived HLA-DR peptides presented by self antigen-presenting cells [17]. We showed that the T cell clones generated from patients with CAN were of the Th1 phenotype, while those generated from stable patients were Th2. In our current study ELISPOT analysis was used first to confirm these results. As shown in Figure 1 T cell clones generated from patients with CAN produced IFN-γ (prototype Th1 cytokine), while those generated from stable patients produced IL-10 (prototype Th2 cytokine) in response to the donor-derived mismatched HLA-DR allopeptide in vitro. Three or four clones with different T cell receptor phenotypes were tested from each of the patients and similar results were obtained in all cases.

We then investigated whether the Th2 clones function to regulate Th1 alloimmune responses. The regulatory functions of Th2 clones have been described in both experimental autoimmune [19, 20] and transplant animal models [17], but have not as of yet been demonstrated in humans. Therefore, a co-culture system was established where Th1 and irradiated (to prevent their proliferation) Th2 clones were incubated in vitro with the relevant HLA-DR allopeptide. Figure 2 shows that the addition of the irradiated Th2 clones significantly inhibited the proliferative response of the Th1 clones (by 57%) to the relevant HLA-DR allopeptide. The irradiated Th2 clones alone had minimal proliferation in response to the HLA-DR allopeptide, ruling out the possibility that the inhibition of proliferation in the co-culture system was due to overcrowding of cells or over-consumption of growth media in culture wells. In addition, inhibition of proliferation did require the presence of the relevant HLA-DR peptide to stimulate the Th2 clones to regulate the Th1 clones in vitro (Fig. 2).

Then the effects of Th1 and Th2 cell interactions on cytokine production were evaluated in vitro using ELISA. Cytokine analysis of co-culture supernatants showed in-

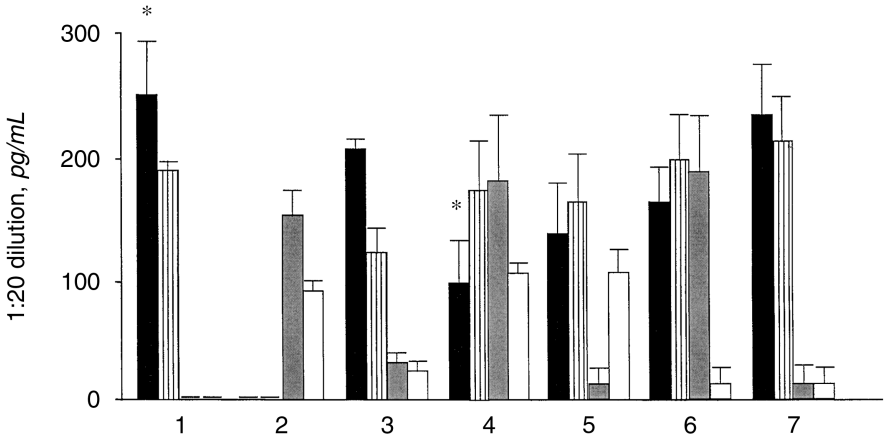

Fig. 3. Regulatory function of Th2 clones. Cytokine profiles of co-culture supernatants of Th2 clones from patients with stable renal function (SRF) irradiated with 3000 rads, cultured in a 1:1 ratio with Th1 clones from chronically rejecting patients (CR). Symbols are: (■) IFN-γ; (▥) IL-2; (▩) IL-4; (□) IL-10. (*1*) Th1 clones specific for HLA-DR1 (42-62) + peptide HLA-DR1 (42-62). (*2*) Irradiated Th2 clones specific for HLA-DR2 (41-60) + peptide HLA-DR2 (41-60). (*3*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptide HLA-DR1 (42-62). (*4*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60). (*5*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing IL-4 mAb. (*6*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing IL-10 mAb. (*7*) Th1 clones specific for HLA-DR1 (42-62) + irradiated Th2 clones specific for HLA-DR2 (41-60) + peptides HLA-DR1 (42-62) and HLA-DR2 (41-60), and neutralizing anti-IL-4 and anti-IL-10 mAb. Data are expressed as mean ± SEM, *Reduction in IFN-γ, 1 vs. 4, $P < 0.0001$, $N = 4$).

hibition of IFN-γ (by 46%) but not of IL-2 production by the Th1 clones in the presence of Th2 clones in response to the relevant HLA-DR peptide (Fig. 3). These data indicate that there is a dissociation between the inhibition of Th1 cell proliferation and IFN-γ production on the one hand and IL-2 secretion on the other. This is an interesting observation. We are uncertain whether this is unique to our indirectly primed T cells clones, and if so, what the mechanism for this would be. Clearly, proliferation is not dependent solely on IL-2 in these clones; however, we are uncertain of the exact relationship of INF-γ and proliferation other than to say that they are both impaired by co-culture with the Th2 clones.

Similar to the proliferation studies described above, addition of Th2 clones with different peptide specificity did not result in inhibition of cytokine production (Fig. 3). As expected, production of the Th2 cytokines IL-4 and IL-10 by the irradiated Th2 clones was preserved and was not affected by co-culture with the Th1 clones (Fig. 3). Although irradiation reduced the ability of Th2 clones to proliferate, irradiated clones were still able to produce IL-4 and IL-10 in response to appropriate antigenic stimulation. As cytokine production and cell proliferation are two different functions of the T cell response to antigen, they may respond differently to irradiation. Previous publications have shown the differential effects of irradiation on the ability of T cells to proliferate and produce cytokines [21]. To further confirm these results and further dissect the mechanisms of regulation, proliferation of and cytokine production by the Th1 co-cultured with Th2 cell clones were measured in the presence or absence of neutralizing anti-IL-4, anti-IL-10 mAb, or isotope-matched control. With the presence of the neutralizing anti-IL-4 mAb the proliferative response of the co-cultured Th1 and Th2 cell clones was enhanced compared to the response in absence of the antibody. However, this response was lower than the normal response of Th1 cell clones alone. A comparable effect was observed when neutralizing anti-IL-10 mAb was added. However, addition of both neutralizing antibodies (anti-IL-4 and anti-IL-10) to co-cultured Th1 and Th2 cell clones fully restored the T cell proliferative response; indeed the proliferative responses were slightly higher than the Th1 clone alone (Fig. 2). Furthermore, as shown in Figure 3, neutralizing anti-IL-4 or anti-IL-10 mAb failed to normalize IFN-γ production by Th1 and Th2 co-cultured cell clones. However, when both of the neutralizing antibodies (anti-IL-4 and anti-IL-10) were added to the Th1 and Th2 co-culture the production of IFN-γ was restored. The control antibodies had little effect (data not shown).

DISCUSSION

Collectively, our data clearly demonstrate, to our knowledge for the first time, a regulatory function of HLA-DR allopeptide-specific Th2 cell clones in humans and

confirm our previous similar findings in an animal model. The Th1 clones in the animal study were derived from acutely rejecting recipients of a renal allograft, while in this study the human Th1 clones were derived from individuals undergoing chronic rejection. This suggests that the biological process of indirect allorecognition in both acute and chronic rejection are similar, but may differ quantitatively rather than qualitatively, with a lower alloreactive T cell clone frequency occurring in chronic rejection. The importance of Th2 clones in tolerant and benign responses to alloantigen has been debated, and evidence in the literature suggesting that deviation to a Th2 phenotype is either a cause or an effect of a tolerant response remains controversial. In an interesting study, Li et al showed that inhibiting Th1 responses by a neutralizing anti-IL-12 antibody was effective in inhibiting alloimmune responses to minor but not major histoincompatibility antigens [22]. The authors suggested that one potential explanation is that Th2 immune deviation may be more effective in inhibiting an indirect alloimmune response where the alloreactive T cell clone size is relatively small. However, data indicating that a Th2 clone can regulate a Th1 alloimmune response in vivo have been lacking. Besides our initial results in animals [17], VanBuskirk and colleagues recently published data indicating that peripheral blood lymphocytes from transplant patients off immunosuppression with stable allograft function can function as regulatory cells whose function may be mediated through IL-10 and transforming growth factor-β (TGF-β) [23]. Our data further support the notion that an indirect Th1 alloimmune response is deleterious and promotes chronic rejection, while an indirect Th2 alloimmune response may be regulatory and protects against chronic rejection and may promote tolerance.

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